METHOD OF ISOLATING CD8$^+$ CELLS, AND RELATED HYBRIDOMA CELLS, ANTIBODIES AND POLYPEPTIDES

B1

Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

Field of the Invention

This invention relates to a positive selection method for isolating CD8$^+$ cells using certain CD8-specific antibodies. The isolated CD8$^+$ cells have importance as vehicles for combating viral infections and tumors.

Background of the Invention

In humans, CD8$^+$ cells play a vital role in the immune system's ability to defend against potentially harmful foreign entities, such as bacteria and viruses [1]. CD8$^+$ cells circulate in the blood and possess on their surface the CD8 protein. When necessary, these cells are converted into cytotoxic cells (i.e. cell-killing cells) which proceed to destroy foreign cells, viruses, and other harmful pathogens present in the subject [2]. Because of CD8$^+$ cells' effective role in host defense, they hold great potential in isolated form as therapeutics for treating disorders such as viral infections and malignancies [3].

In the past, purification of human CD8$^+$ cells has been achieved by negative selection. Specifically,

peripheral blood mononuclear cells ("PBMC's") are incubated with a cocktail of monoclonal antibodies specific for non-CD8 sub-populations. These sub-populations include, for example, B-cells, $CD4^+$ cells, NK cells, macrophages and neutrophils, and each contains specific, non-CD8 "markers". The sub-populations ar then removed from the resulting antibody cocktail using magnetic beads [4]. This technique has certain major disadvantages. The first is that several monoclonal antibodies are required for removing non-$CD8^+$ cells. The second is that the resulting $CD8^+$ population suffers from contamination from non-$CD8^+$ cells that possess relatively low levels of non-CD8 markers. Finally, when a magnetic separation procedure is used to remove all non-$CD8^+$ cells, a large number of magnetic beads are needed.

Summary of the Invention

This invention provides a method of isolating $CD8^+$ cells which comprises the steps of

(a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTQRFSG, (SEQ ID NO:1) or portion thereof, on CD8 molecules present on the surface of $CD8^+$ cells but does not activate the $CD8^+$ cells once bound thereto, under conditions permitting the formation of a first complex between the $CD8^+$ cell and first antibody;

(b) separating from the sample any first antibody not present in the resulting first complex;

(c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the $CD8^+$ cells present in the sample;

(d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);

(e) contacting the immobilized second complex under suitable conditions with an agent which caus s the dissociation of the second complex into $CD8^+$ cells and an immobilized third complex between the first antibody and second antibody; and

(f) separating the immobilized third complex from the $CD8^+$ cells, thereby isolating the $CD8^+$ cells.

This invention also provides a hybridoma cell line which produces a monoclonal antibody which specifically binds to CD8 molecules present on the surface of $CD8^{+}$ cells but does not activate the $CD8^{+}$ cells. This invention further provides monoclonal antibodies produced by each of the instant hybridoma cell lines. Finally, this invention provides related polypeptides, isolated $CD8^{+}$ cells and kits.

Detailed Description of the Invention

The hybridoma cell lines designated 37B1 and 8G6 were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 2010-2209 under ATCC Accession Nos. HB-12441 and HB-12657, respectively.

This invention provides a method of isolating $CD8^+$ cells by employing an anti-CD8 antibody, along with certain other reagents. Specifically, this invention provides a method of isolating $CD8^+$ cells which comprises the steps of

(a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTQRFSG, (SEQ ID NO:1) or portion thereof, on CD8 molecules present on the surface of $CD8^+$ cells but does not activate the $CD8^+$ cells once bound thereto, under conditions permitting the formation of a first complex between the $CD8^+$ cell and first antibody;

(b) separating from the sample any first antibody not present in the resulting first complex;

(c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the $CD8^+$ cells present in the sample;

(d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);

(e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into $CD8^+$ cells and an immobilized third complex between the first antibody and second antibody; and

(f) separating the immobilized third complex from the $CD8^+$ cells, thereby isolating the $CD8^+$ cells.

As used herein, a "$CD8^+$ cell" means a T-cell having on its surface the CD8 protein. In the preferred embodiment, the $CD8^+$ cells are human $CD8^+$ cells. The $CD8^+$ cells can be from any $CD8^+$ cell-possessing species. "Isolating" $CD8^+$ cells means obtaining a population of peripheral mononuclear blood cells wherein the ratio of $CD8^+$ cells to non-$CD8^+$ cells is at least about 7:1. In the preferred embodiment of this invention, this ratio is at least about 9:1.

Sub C1

This invention employs several types of antibodies which specifically bind to given epitopes. More particularly, this invention uses a "first antibody" which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of $CD8^+$ cells but does not activate the $CD8^+$ cells once bound thereto. Here, $CD8^+$ cell "activation" means causing $CD8^+$ cells to express γ-interferon ("γ-IFN"). This activation can be measured using routine methods such as sandwich ELISA assays, which can be performed using commercially available kits.

Such first antibodies include, but are not limited to, the monoclonal antibodies produced by the hybridoma cell lines 37B1 (ATCC Accession No. HB-12441) and 8G6 (ATCC Accession No. HB-12657). Conditions which permit these antibodies to bind to but not activate $CD8^+$ cells are well known in the art. These conditions are described, for example, in a suitable buffer such as $Ca2^+$ and $Mg2^+$-free Dulbecco's Phosphate Buffer Saline (DPBS) containing 1% Human Serum Albumin (HSA) and 0.2% sodium citrate and gentle mixing by "end over end" rotation on a rotator set at 4 rpm.

Sub B3

As used herein, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. In one embodiment, the antibody is a monoclonal antibody. The monoclonal antibody can be human, or that of another species including, for example, mouse and rabbit. In this invention, an antibody which "specifically" binds to a stated epitope binds to that epitope with a dissociation constant of at least about 10-fold less than the dissociation constant with which it binds to any other epitope. In one embodiment, this dissociation constant ratio is at least about 100. In the preferred embodiment, this dissociation constant ratio is at least about 10^3.

The "second antibody" used in the instant method can be any antibody which specifically recognizes an epitope on any portion of the first antibody. In the preferred embodiment, the second antibody specifically recognizes a

portion of the constant (Fc) region of the first antibody. Such anti-Fc antibodies are commercially available and include, for example, sheep anti-mouse antibody immobilized on magnetic beads [5].

Sub C2

The agent that causes dissociation of the immobilized second complex into $CD8^+$ cells and immobilized antibodies can be any agent which successfully competes with the CD8 molecule for specific binding to the first antibody. In the preferred embodiment, this agent is the polypeptide designated CD8-3 having the sequence AAEGLDTQRFSG. In one embodiment, the immobilized second antibody comprises an antibody operably affixed to a magnetic bead.

This invention also provides a hybridoma cell line which produces a monoclonal antibody which specifically binds to CD8 molecules present on the surface of $CD8^+$ cells but does not activate the $CD8^+$ cells. In one embodiment, the hybridoma cell line is selected from the cell lines designated 37B1 (ATCC Accession No. HB-12441) and 8G6 (ATCC Accession No. HB-12657). This invention further provides the monoclonal antibodies produced by each of the instant hybridoma cell lines.

Sub C3

This invention further provides a polypeptide useful for generating the instant monoclonal antibody that comprises the amino acid sequence AAEGLDTQRFSG. In the preferred embodiment, the polypeptide is the polypeptide designated CD8-3 and having the amino acid sequence AAEGLDTQRFS. The instant polypeptide can optionally comprise one or more additional amino acid residues at the C-terminal or N-terminal end. In the preferred embodiment, the polypeptide has the sequence NKPKAAEGLDTQRFSGKRLG.

This invention further provides a population of $CD8^+$ cells isolated by the instant method.

Sub C4

Finally, this invention provides a kit for use in isolating $CD8^+$ cells which comprises, in separate compartments, (a) an antibody which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of $CD8^+$ cells, but does not activate the $CD8^+$ cells once bound thereto; and (b) an agent which causes the dissociation of a $CD8^+$ cell-antibody complex. In one embodiment, the agent which causes the dissociation of a $CD8^+$ cell-antibody complex comprises the polypeptide having the sequence AAEGLDTQRFSG. In the preferred embodiment, the agent is the polypeptide consisting of the sequence AAEGLDTQRFSG.

The instant kit can further comprise reagents useful for performing the binding and dissociation steps of the instant method. The components of the instant kit can either be obtained commercially or made according to well known methods in the art. In addition, the components of the instant kit can be in solution or lyophilized as appropriate. In the preferred embodiment, the kit further comprises instructions for use.

The following procedures relating to the instant invention are routine in the art: isolating peripheral mononuclear blood cells from which the $CD8^+$ cells are in turn isolated [6]; separating unbound antibodies and cells from a sample containing bound antibodies and/or cells via centrifugation or spinning membrane; and immobilizing antibodies via polystyrene flasks, columns or beads [4,7].

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Rationale

Human $CD8^+$ cells can be isolated from preparations of peripheral blood mononuclear cells (PBMC's) by either positive or negative selection. Positive selection results in a highly-purified population of $CD8^+$ cells. Negative selection, while resulting in sufficient numbers of $CD8^+$ cells, has low levels of contaminating non-CD8 populations remaining after the selection procedure.

The idea was to generate an antibody which has high affinity for $CD8^+$ cells, does not activate the cells during the selection process, and is capable of being easily eluted from the cells. An anti-peptide antibody appeared to meet these criteria. However, it was known that anti-peptide antibodies might be of low affinity and may recognize the linear peptide sequence exclusively, preventing reactivity with native antigen.

It was necessary that the anti-CD8 antibody not activate the cells during the selection process, as it would lessen their ability to effectively act as naïve responder cells during *in vitro* stimulation protocols. The use of peptide release to selectively isolate a cell population has been show by Tseng-Law, et al. [8] for $CD34^+$ cells.

Methods

The CD8 alpha chain was examined for hydrophilic sequences and four peptides selected. All were coupled to keyhole limpet hemocyanin (KLH) as carrier and used to immunize mice. A C-terminal amino acid was added to each of the peptides coupled to KLH to make the monoclonal antibodies. Antisera from the mice were evaluated for the ability to recognize both peptide and native CD8 on the surface of T cells. Only one of the four peptides was capable of recognition of both antigenic forms of CD8. Monoclonal antibodies were generated to this peptide and the resulting antibody used to isolate $CD8^+$ cells from a PBMC preparation. The antibody was successful in isolating a population of highly-purified $CD8^+$ cells (Table 1) which were not activated by the isolation procedure (Table 2).

Table 1

Purification of $CD8^+$ Cells by Positive Selection Analyzed by Flow Cytometry*

CELL TYPE	PE/PC Fluorescence (Range)	Post Selection FITC Fluorescence (Range)
CD8 T cells	15 (7-24)	82 (56-95)
CD4 T cells	36 (14-52)	2 (0.1-10)
CD14 Monocytes	15 (7-26)	0.8 (0.2-2)
CD15 Neutrophils	12 (8-21)	0.6 (0.1-3)
CD19 B cells	2 (0.4-7)	3 (0.5-9)
CD56 NK cells	6 (2-17)	6 (0.1-20)

* Summary of 10 normal donors

Table 2

Activation of $CD8^+$ Cells Isolated By Negative or Positive Selection (Assessed by IFNγ Production)

Cells	Negative Selection (pg/ml)	Positive Selection (pg/ml)
un-stimulated	20	20
allo-stimulation	1440	3600

References

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